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13. ABSTRACT (Maximum 200 words)

The ultimate goal of the project is to synthesize three specific peptides (cercropin P1, cercropin A and PGQ) in order to determine their anti-microbial activity. To expedite this goal, mass spectrometry was used to identify products of the solid phase synthesis and thereby optimize synthetic conditions. Three different analytical approaches – MALDI-TOF, LC-electrospray ionization (ESI) ion trap MS and direct infusion ESI Ion Trap MS – were examined. Of particular consideration was the selection of methodology compatible with instrumentation capabilities at Soldier Systems Laboratory, Natick, MA for eventual incorporation of the techniques in-house. Direct infusion ESI Ion Trap MS provided the best results and most realistic analytical approach for purposes of this program given the instrumentation available. . .

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Enclosure 1

Solid Phase Peptide Synthesis of Antimicrobial Peptides for Cell Binding Studies: Characterization Using Mass Spectrometry

Objectives

This project will attempt to increase the anti-microbial activity of three specific peptides (cercropin P1, cercropin A, and PGQ). In order to expedite this goal, mass spectrometry will be used to identify products of the synthesis of these peptides and hereby optimize synthesis conditions.

Introduction

Anti-Microbial peptides have been known for their ability to bind to cell membranes. If certain peptides were proven to selectively bind to target cells, these peptides could have applications ranging from tagging cells for easy detection to disrupting cellular activity, which would ultimately lead to cell death.

In order for these peptides to have practical applications, they must bind efficiently to the target cell. Although it can be demonstrated that different peptides have a wide range of binding efficiencies for a particular cell, it has not been determined what differences in these peptides are responsible for the binding efficiency. If it could be proven that specific peptide properties (such as length or amino acid sequence) influenced the binding efficiency to a particular cell, these properties could be exploited in order to create a more efficiently binding peptide.

One integral phase of this research project is the quantitative and qualitative analysis of the peptide synthesis process. The synthesis process needs to be monitored to determine the concentration and identity of all produced compounds. It is rare that a solid phase synthesis will produce a high percent yield of the desired product without any byproducts. Thus, if the crude products generated from the synthesis were used in binding assays, one could not determine if the binding properties observed are due to the peptide, impurities or a combination of both. Therefore, it is crucial to establish a reliable method for assessing the integrity of the synthetic process, on complex systems, in a timely fashion.

The most obvious choice for detection of the synthesis products would be mass spectrometry (MS). Although most all modes of mass spectrometry provide mass information, there are many fundamentally different MS systems available that have many advantages and disadvantages. While MS systems such as Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) demonstrate extremely high sample throughput, it does not provide as much structural information as the lower throughput Electrospray ion trap.

This project was an attempt to develop a detection method that could provide information that would allow for optimization of peptide synthesis. Once identified, this method would be optimized for reproducibility, robustness and throughput. Accordingly, a series of solid phase synthesis reactions that generated a diverse number of peptides were used as models in order to establish the appropriate analytical method for their

characterization. These samples were evaluated for their integrity using three approaches; MALDI, direct infusion ESI-MS, and LC-MS-ESI. In comparing the results, it should be borne in mind that the three approaches are representative of the specific MS systems available to the investigators. While some equipment, in particular the MALDI system, may not be fully commensurate with state of the art instrumentation, this is nonetheless a realistic situation.

Peptide Synthesis

All synthesis was conducted in an Advanced Chemtech 396Ω. Peptide synthesis took place on a Wang resin. Each resin was purchased with the first amino acid coupled to the resin. The 396Ω was equipped with a reaction chamber with 40 separate reaction wells. A Styrofoam frit was fit into each of the wells. Table 1 shows the name of every synthesized peptide, the reaction chamber the synthesis took place in, the amino acid sequence of the peptide, the amount of Wang resin used, and the resin binding capacity. All of the protected amino acids were weighed into falcon tubes and diluted with 0.5M N-Hydroxybenzotriazole monohydrate (HOBT) in 1-methyl-2-pyrrolidone (NMP). At this point, the reaction chamber and the amino acid solutions were transferred into the 396Ω. Stock solutions of 25% piperidine in N,N-Dimethylformamide (DMF) and 0.5 M Dichlorobenzoic acid (DIC) in NMP were transferred into the solvent containers within the 396Ω. The 396Ω software package allowed for automated delivery of all amino acids and solvents. Piperidine was delivered to all of the chambers to deprotect the first amino acid bound to the resin. The reaction chambers were then dried down under vacuum. The coupling solvent (DIC in NMP) was delivered to all chambers. The software

package then instructed the 396Ω to deliver the second amino acid in the peptide sequence to each appropriate well. Subsequently, the chamber was gyrated for 30 minutes and the wells were dried again. This coupling step was repeated twice for every amino acid to improve coupling efficiency. (Note. The deprotection step was not repeated thus preventing an undesired double coupling.) The deprotection and coupling steps were repeated for every amino acid in the peptide sequence. A final deprotection cycle was run after the coupling of the last amino acid in order to remove the final protecting group. After this deprotection, the wells were once again dried down under vacuum and the resins transferred to plastic vials. Reagent K was prepared by mixing 2.41 grams of phenol, 1.6 mL of water, 1.6 mL thioanisol and 0.80 mL of ethanediol. 87 uL of reagent K and 423 uL of TFA were added to all resin vials in order to cleave the peptides. After 2 hours the vials were spun down in a centrifuge for 10 seconds at 5000 rpm. (Note. Peptides containing methionine were spun down after one hour) The solvent was removed from the insoluble resin with a disposable pipette and pipetted directly into a falcon tube containing ether that had been chilled to -50 C°. These falcon tubes were stored at -50 C° for 1 hour. The solution was then centrifuged for 10 minutes at 15,844 xg. The peptide formed a white insoluble paste at the bottom of the tube and the ether removed under vacuum. This ether wash process was repeated a total of three times, after which the peptides were reconstituted in 1 mL 0.1% TFA and 1 mL 0.1 % Acetonitrile. The following mass spectrometry procedures were performed on a library of peptides that had been previously synthesized under the direction of Charlene Mello.

MADLI TOF

A matrix solution was prepared by mixing 0.13g sinapinic acid, 0.33 mL 4% TFA, 0.39 mL Acetonitrile and 0.58 mL water. 40 uL of the peptide sample was added to 1.6 mL of the matrix solution. This solution was spotted on the MALDI plate and was allowed to dry for 10 minutes.

Direct Infusion ESI Ion Trap

Stock peptide solutions were diluted 1:100 with a mass spectrometry compatible solvent (10% Acetonitrile, 1%Trifluoroacetic Acid in deionized water) and directly infused into the mass spectrometer (LCQ Thermo Finnegan) at 10 uL per minute using a 250uL Hamilton syringe. Collision Induced Dissociation (CID) was performed between 30% and 50% energy.

LC-ESI-MS

Separation was performed on a Waters Symmetry® C8 column (3.5μm, 2.1 x 150mm). 0.1%TFA in acetonitrile was used as the organic mobile phase. 25uL of the peptide (1:100 dilution) was injected on column. A 40% linear gradient was reached at 40 minutes.

Results

A library of 32 peptide samples was synthesized using the procedure previously described and analyzed by direct infusion using an ESI Ion trap. When analyzed with the ion trap, 9 of the 32 peptide samples showed peaks that would indicate the presence of the expected peptide. The expected masses and ESI Ion Trap data are summarized in Table 2. The signal intensity reflects the ion count as displayed by the mass spectrometer.

As a representative example, Samples 10, 12 and 17 were selected at random for analysis by both MALDI-TOF and LC/MS in addition to direct infusion ESI-MS. This was done to evaluate each method of detection. MALDI-TOF of Sample 10 (Figure 1) showed no evidence of peptides in the sample. Figure 2 shows a spectrum obtained by direct infusion of Sample 10 into the ESI Ion trap. The ions seen in this figure are not representative of a peptide. The peaks are separated by 126 mass units which indicates the likely presence of a polymer or cluster, most likely clusters of TFA and Na^+ ions. The absence of peptides in Sample 10 was also verified in the LC-MS analysis of the sample (Figure 3) which displays a trace for all masses between 1217.5 amu and 1218.5 amu as only background noise throughout the separation.

Sample 17 showed no evidence of masses indicative of peptides in either MALDI-TOF or direct infusion analysis (Figures 4 and 5 respectively). This data was further confirmed with the extracted ion chromatogram for masses between 1205 amu and 1206 amu (Figure 6). This chromatogram shows that these peaks are related to background.

The MALDI-TOF mass spectrum of Sample 12 (Figure 7) shows peaks at m/z 1268.24 and m/z 1506.61 The peak at m/z 1268 is within nearly 2 Da of the protonated peptide. The identity of the peak at m/z 1506 cannot be determined since this particular MALDI-TOF cannot provide structural information. Also, this mass spectrum cannot provide information for compounds below m/z 700 because of the high noise level in that mass range. Figure 8 shows the spectrum obtained by direct infusion of Sample 12 into an

ESI-Ion trap. The peak at m/z 1266.6 corresponds to the protonated peptide, with the corresponding sodium adduct observed at m/z 1288. The peaks at m/z 634 and m/z 667 represent the doubly charged ions of m/z 1266 and m/z 1288. Figure 9 shows the total ion chromatogram from the LC/MS of Sample 12. The mass spectrum for the peak at 12.48 minutes is shown in the Figure inset reflects the presence of the peptide in Sample 12. The extracted ion chromatogram (m/z 1266.0-1267.0) for this same LC/MS run (Figure 10), clearly identifies the integrity of the sample.

In effect, the examination of Samples 10, 12 and 17 verified that all three methods of detections produced comparable results.

Discussion

The project goal was to develop a suitable, rapid analytical method to allow for the optimization of synthesis conditions. Due to time constraints, it was decided that LC/MS of all peptides would not be practical for initial screenings as it is too time consuming and was used here in order to validate results obtained by direct infusion ESI Ion trap and MALDI-TOF. While in terms of throughput, MALDI-TOF was the better choice, the deficiencies associated with the particular system available at Natick Soldier System Labs, render the approach inadequate for this project. A more modern system equipped with a reflectron analyzer would provide much better resolution and better mass accuracy. Additionally, new systems are capable of performing collision induced dissociation (CID), which would also provide structural information.

Specifically, MALDI-TOF was by far the quickest method of detection. It took approximately 1 hour to prepare 30 samples for MALDI-TOF and then between 1 and 5 minutes to obtain molecular mass information for each peptide. This is advantageous when there is a need for high throughput screening of a large number of samples, but, as stated above, the particular instrument used here did not have sufficient mass accuracy. MALDI-TOF also suffers from a poor sensitivity at the lower mass range (< 450 amu). Although all of the peptides in this particular library have masses well above 450 amu, obtaining mass information below the molecular mass region of the peptide is crucial in order to confirm low molecular weight by-products.

Data obtained by direct infusion ESI ion trap MS agreed with both LC/MS and calculated mass assignments as illustrated in the example of the analysis of Sample 12. In addition, the electrospray spectra obtained by direct infusion revealed the presence of other peptides even without separation. The identities of these compounds were established with MS/MS.

Direct infusion ESI Ion Trap took anywhere from 1 hour to 1.5 days per sample depending on its complexity. Finally, assuming similar response and ionization efficiencies , the direct infusion analysis was also able to provide some semi-quantitative data on the peptides of different nominal mass by comparing the ion count of each analyte.

The value of ESI ion trap analysis to provide structural data is illustrated using as an example, the peptide in Sample 30 (MW 1138). While MALDI-TOF can confirm the molecular mass of the compound (data not shown), the collision induced dissociation (CID) spectra of the protonated molecule, Figure 11, provided valuable information about its structure. The peak at m/z 1120 represents the loss of the C-terminal hydroxyl group. The peak at m/z 1007 represents the peptide after the first amino acid (leucine) has been cleaved off, thus showing a loss of 131 amu. Figure 11 shows peaks that represent the cleavage of the first 6 amino acids on Sample 30. In order to confirm the complete amino acid sequence, MS/MS can be performed after the first 6 couplings steps of the synthesis. This data can help to determine which coupling steps failed, whereas the MALDI-TOF would only indicate what masses are present.

Summary and Recommendations

We have evaluated three different analytical approaches, MALDI-TOF, LC- ESI ion trap MS and direct infusion ESI ion trap MS, for the characterization of peptides produced by solid phase synthesis in order to streamline the production and testing of the anti microbial activity of the related peptides. Given the instrumentation available both at Northeastern and the Natick labs, we have identified direct infusion ESI ion trap MS as the most realistic method of analysis for the purpose of this program. The data obtained suggested that infusion ESI ion trap MS represents a reasonable compromise in terms of reliability and throughput which will allow the project to proceed without hindrance.

Table 1.

This table shows the well that the reaction took place in, the name and sequence of the peptide, the binding capacity and the mass of resin used.

Reaction Well	Peptide	Amino Acid Sequence	Resin capacity (mmol/g)	Grams of resin needed
1	CA-4	lgkklegvgkr	0.35	0.100000
2	CP1_3	iaiaiqggpr	0.35	0.100000
3	CP1_4	ktakklensakkr	0.35	0.100000
4	CTH_1	rglrrlgr	0.35	0.100000
5	CTH_5	kkygptylr	0.35	0.100000
6	PGQ_3	vigylkklgt	0.5	0.070000
7	PL_2	gkhvgkaalt	0.5	0.070000
8	CA_3	pvavgikalg	0.6	0.058333
9	CA-5	vfkalpvavg	0.6	0.058333
10	CP1_2	ensakkriseg	0.6	0.058333
11	CPF_3	lgkalkaalkig	0.6	0.058333
12	CTA_4	lkkalpvakkig	0.6	0.058333
13	CTH_2	kiahgvkkyg	0.6	0.058333
14	CTH_3	ptylriiriag	0.6	0.058333
15	CPF_4	anmlggtp	0.7	0.050000
16	CTA_3	piakaalp	0.7	0.050000
17	CTA_5	kialpiakaalp	0.7	0.050000
18	CTH_4	lgrkiahgv	0.8	0.043750
19	PGQ_2	kklgtgalnav	0.8	0.043750
20	PL_1	gwgsffkkaahv	0.8	0.043750
21	PL_3	fkkaahvgkhv	0.8	0.043750
22	CP1-5	isegiaiaiq	0.8	0.043750
23	PGQ_4	galnavlkq	0.8	0.043750
24	CA_1	gglkklgkkl	0.9	0.038889
25	CA-2	egvgkrvfkal	0.9	0.038889
26	CP1_1	swlssktakkl	0.9	0.038889
27	CPF_1	gfasflgkal	0.9	0.038889
28	CPF_2	kaalkiganml	0.9	0.038889
29	CTA_1	sigsalkkal	0.9	0.038889
30	CTA-2	pvakkigkial	0.9	0.038889
31	PGQ_1	gvlsnvigyl	0.9	0.038889
32	PL_4	gkaalthyl	0.9	0.038889

Table 2. AMPFRAG Below is a summary of the Mass Spectra obtained from direct infusion ESI-MS. The signal intensity reflects the total ion count. N/A indicates that these peptides were not synthesized. TFA indicates that all major peaks were due to Trifluoroacetic acid.

Peptide Number	Peptide	Sequence	Calculated Mass	Major Peak	Signal Intensity	Second Peak
1	CA-4	lgkklegvgkr	1184.42	719	5.00E+03	
2	CP1_3	[illegible]	995.16	TFA		
3	CP1_4	ktakklensakkr	1501.73	734, 1055	3 E 3	
4	CTH_1	[illegible]	983	TFA		
5	CTH_5	kkygptylr	1125	N/A		
6	PGQ_3	[illegible]	1091.24	N/A		
7	PL_2	gkhvgkaalt	981.13	982	3 E5	1083
8	CA_3	[illegible]	924.12	925	6 E 4	
9	CA-5	vfkalpvavg	1000.21	1001	8 E 4	
10	CP1_2	[illegible]	1218.28	TFA	5 E 3	
11	CPF_3	lgkalkaalkig	1182.48	1183	5 E 5	
12	CTA_4	[illegible]	1265.61	1266	7 E 5	
13	CTH_2	kiahgvkkyg	1101	N/A		
14	CTH_3	[illegible]	1272.42	N/A		
15	CPF_4	anmlggtp	759.85	797	1 E 4	
16	CTA_3	[illegible]	779.94	TFA	2 E 4	
17	CTA_5	kialpiakaalp	1206	TFA	3 E4	
18	CTH_4	[illegible]	950.13	1203	9 E 5	933.2
19	PGQ_2	kklgtgalnav	1071.25	1072	3 E5	1314
20	PL_1	[illegible]	1334.49	TFA	1 E 3	
21	PL_3	fkkaahvgkhv	1221.43	TFA	2 E 5	
22	CP1_5	[illegible]	936.038	1015	2 E 5	
23	PGQ_4	galnavlkq	913.06	915	3 E4	1156
24	CA-1	[illegible]	1041.33	1042	3E4	
25	CA-2	egvgkrvfkal	1203.41	1456	E4	1204
26	CP1_1	[illegible]	1248.44	N/A		
27	CPF_1	gfasflgkal	1010.16	N/A		
28	CPF_2	[illegible]	1129.39	1152	2 E 5	798
29	CTA_1	sigsalkkal	987.17	988	2 E 5	
30	CTA2	[illegible]	1038	1041	7 E 5	
31	PGQ_1	gvlsnvigyl	1034.1	N/A		
32	PL_4	[illegible]	973.01	N/A		

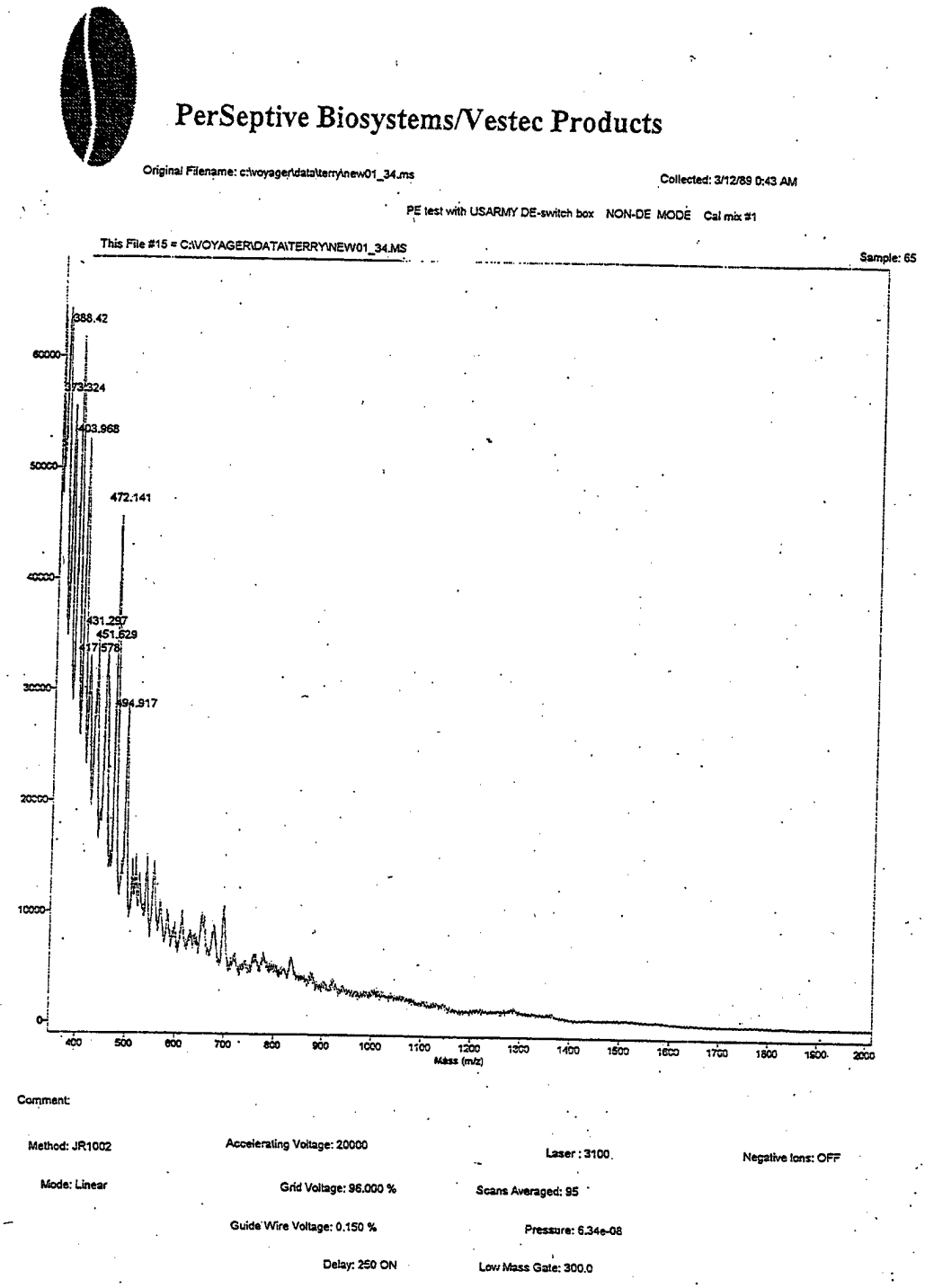

Figure 1.

MADLI-TOF of Sample 10.

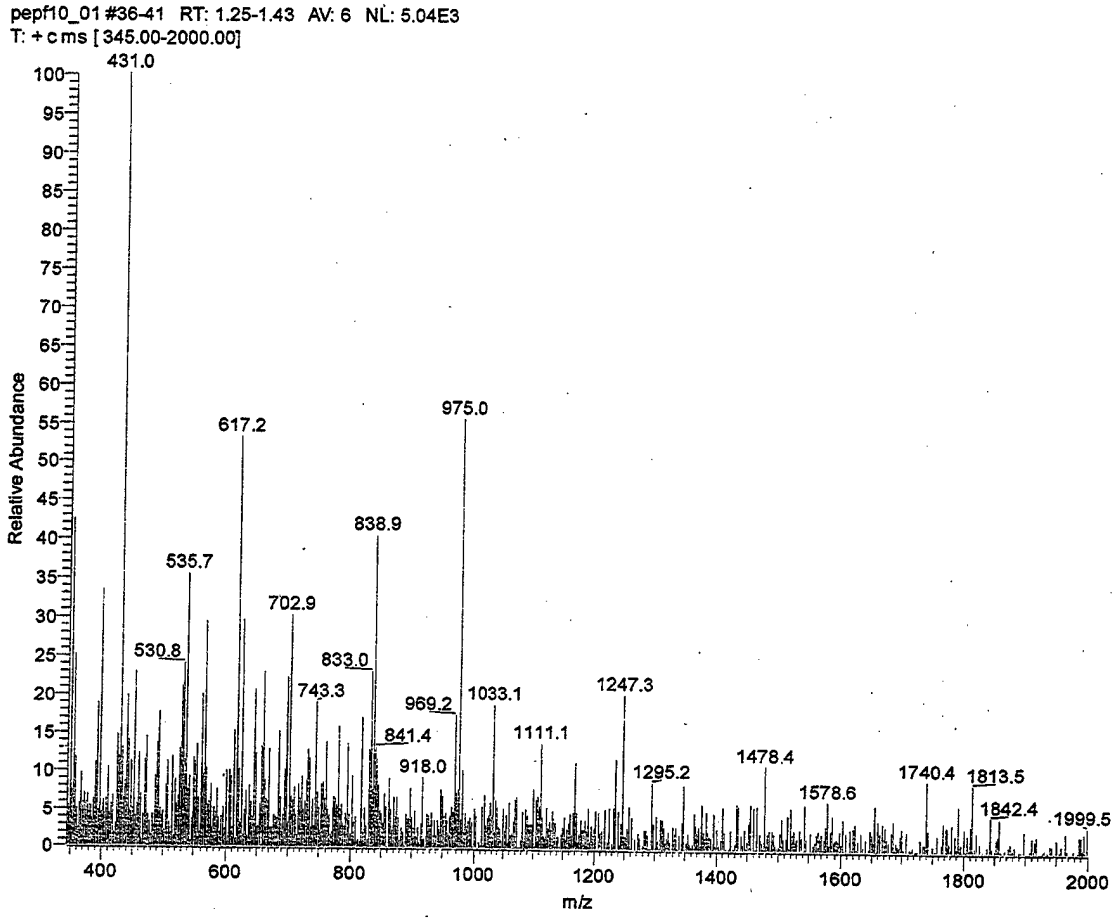

Figure 2. Direct infusion ESI Ion Trap of Sample 10

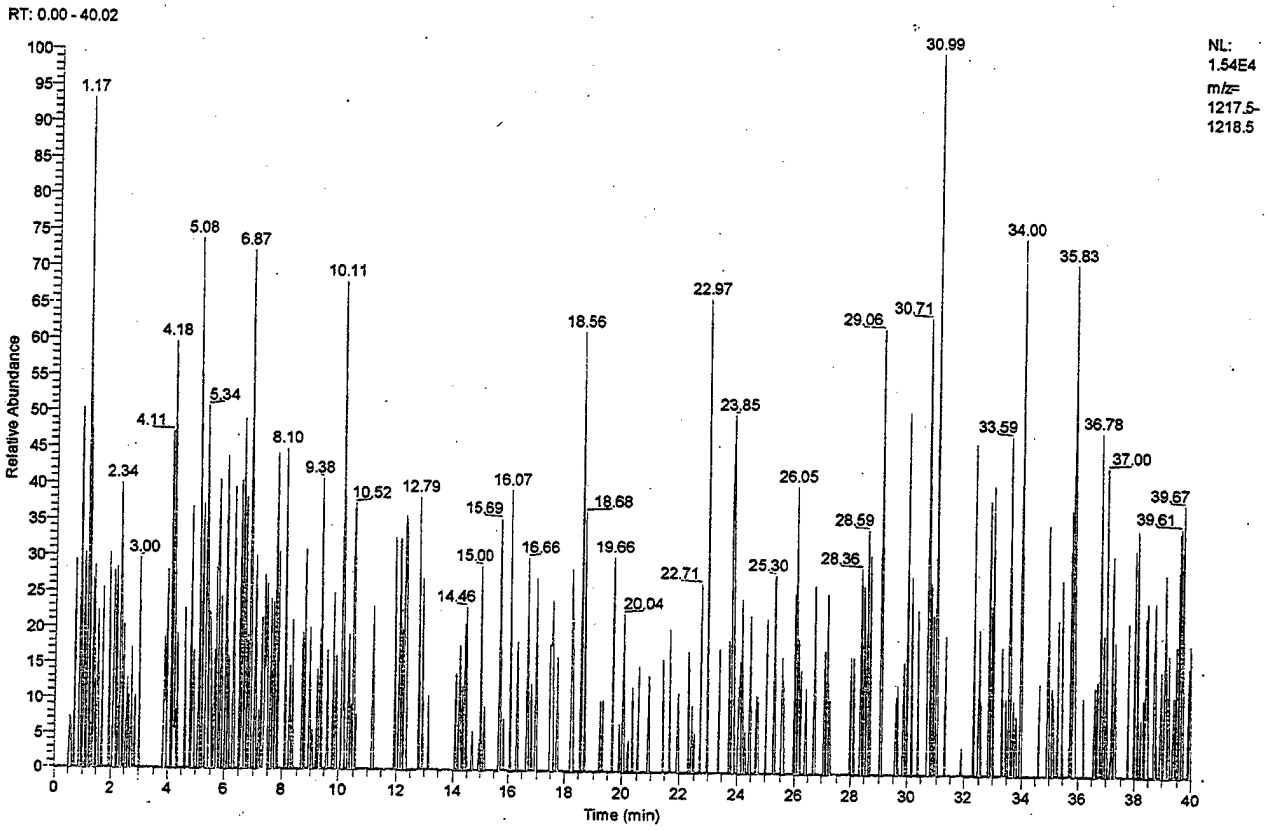

Figure 3. Extracted Ion Chromatogram for Sample 10.

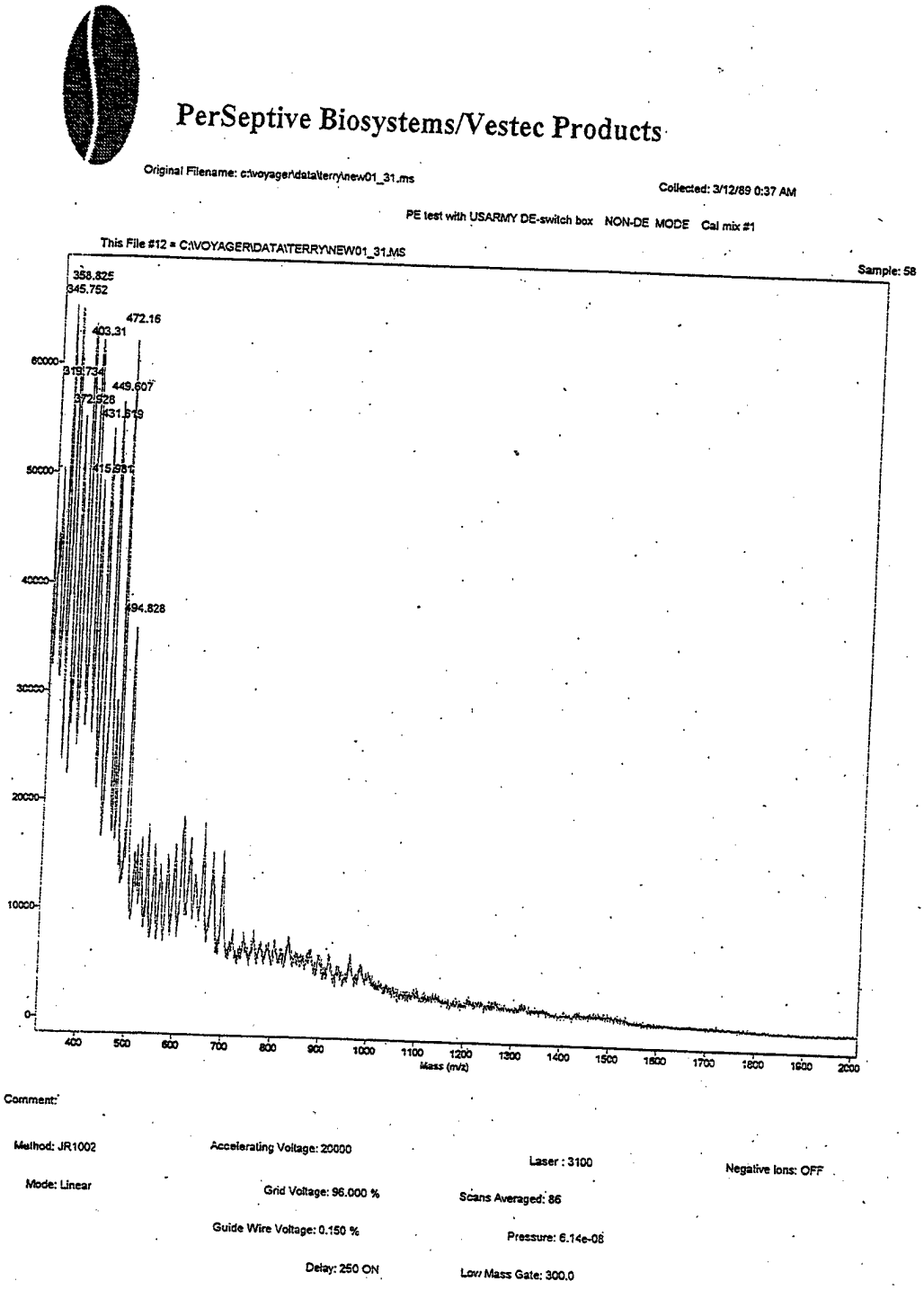

Figure 4.

MADLI-TOF of Sample 17

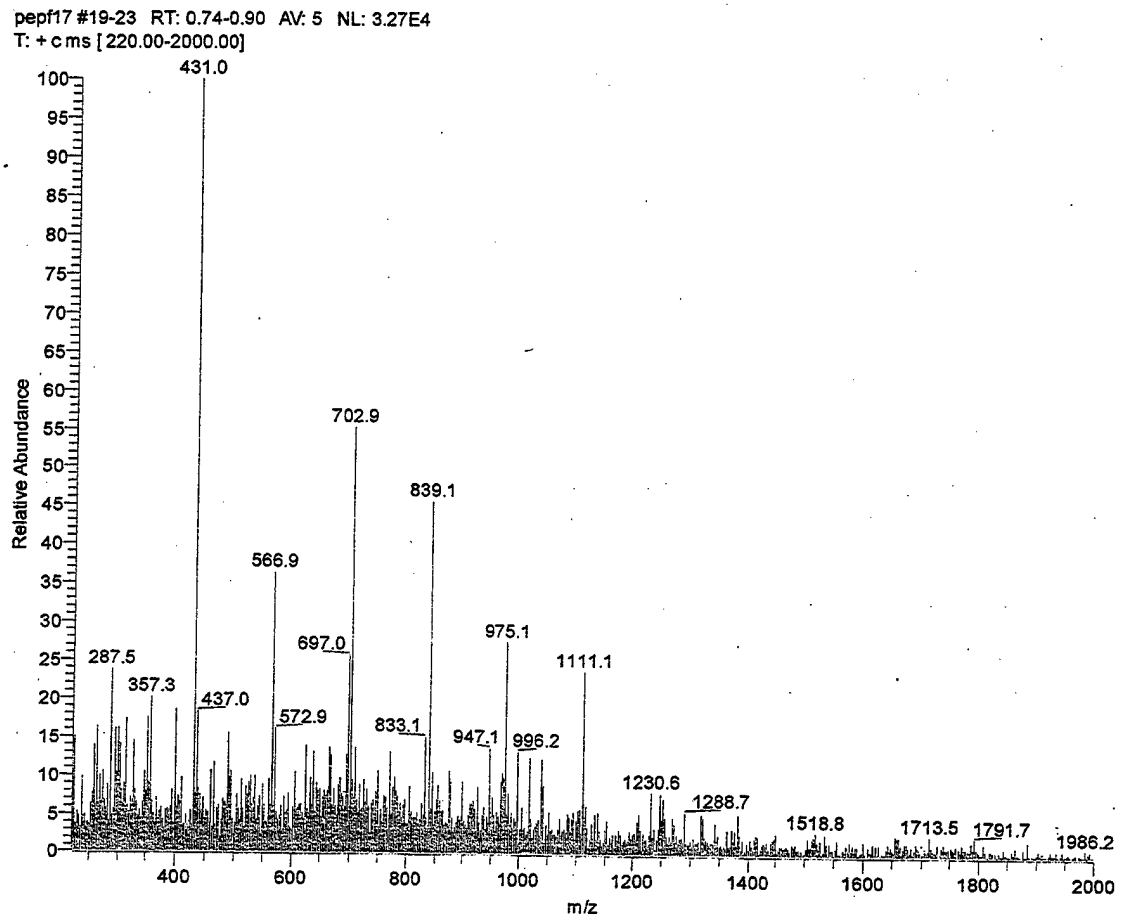

Figure 5. Direct Infusion of Sample 17.

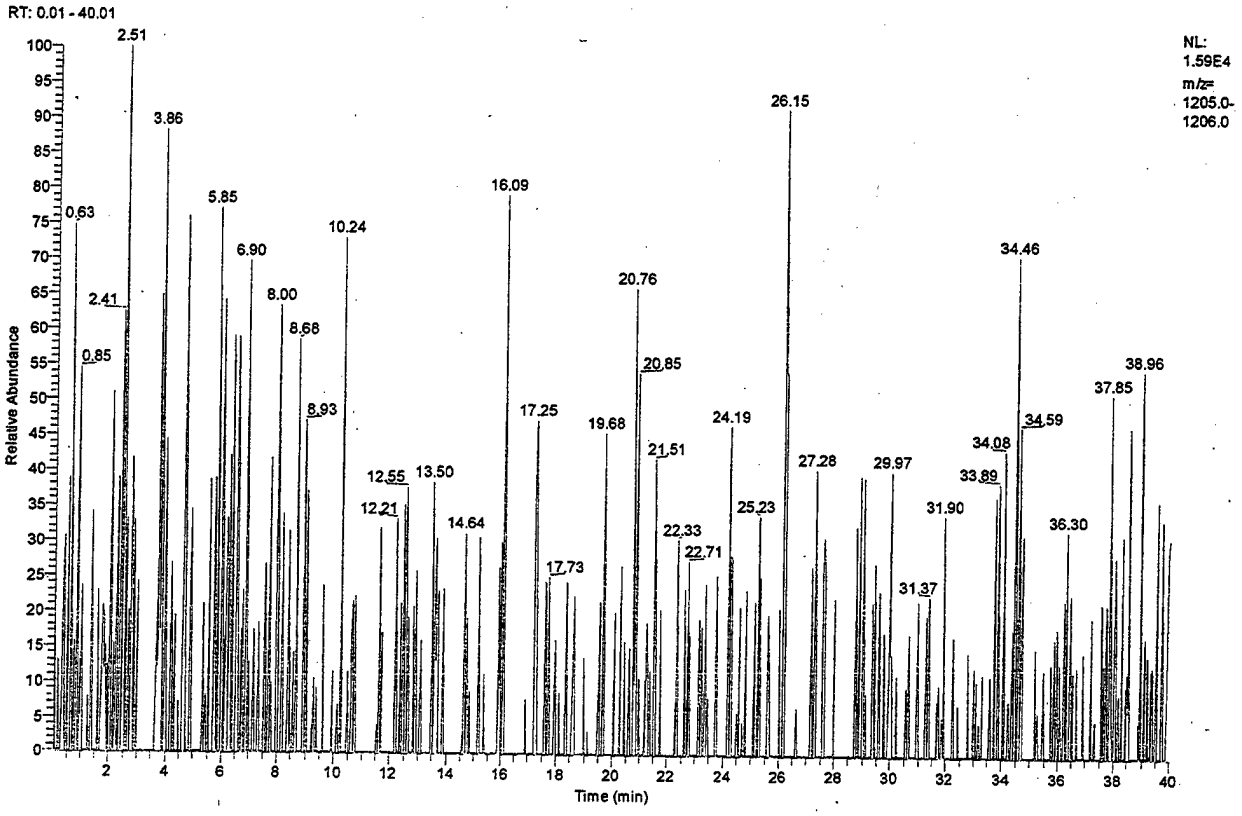

Figure 6. Extracted Ion Chromatogram for Sample 17.

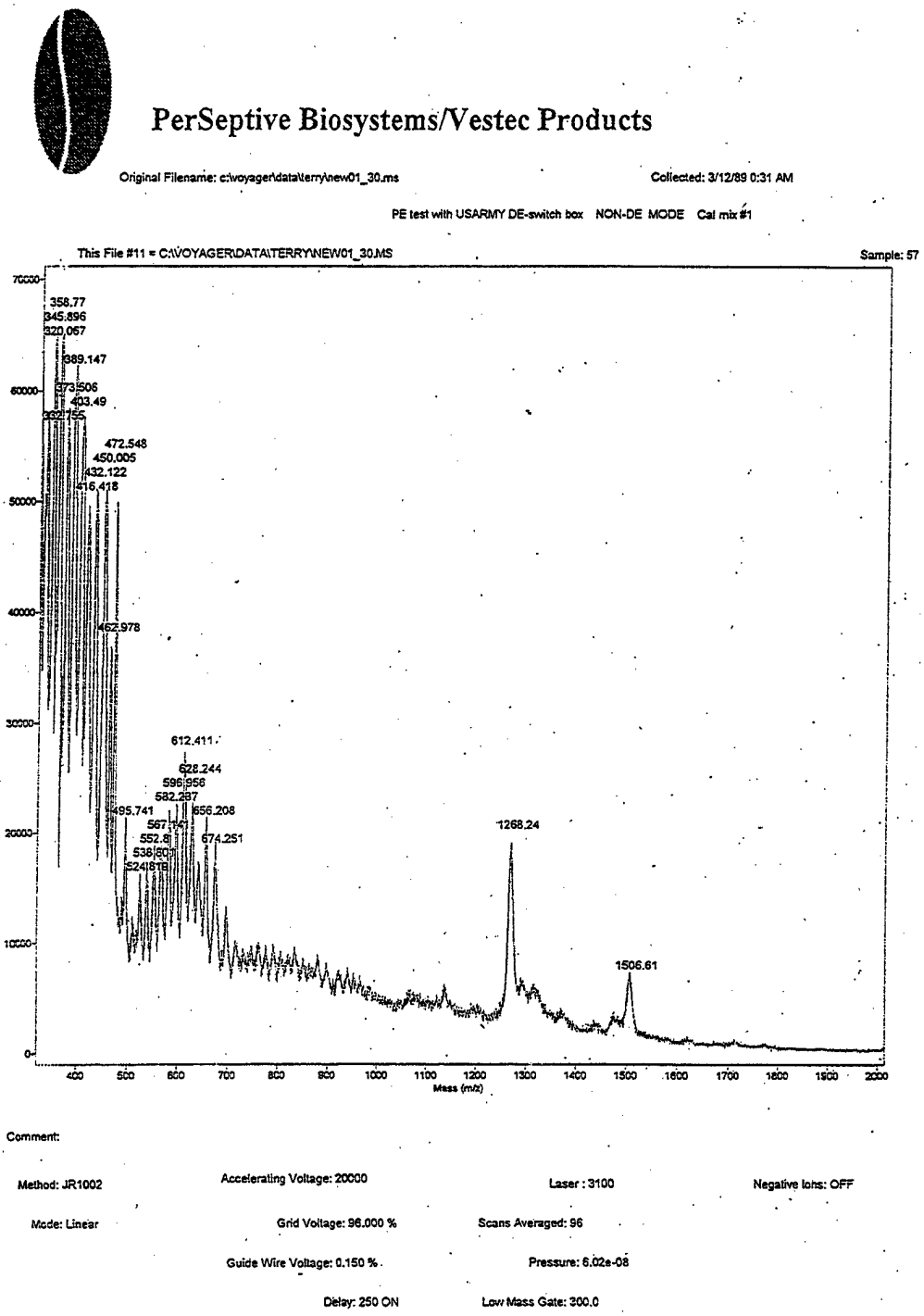

Figure 7. MALDI- TOF for sample 12.

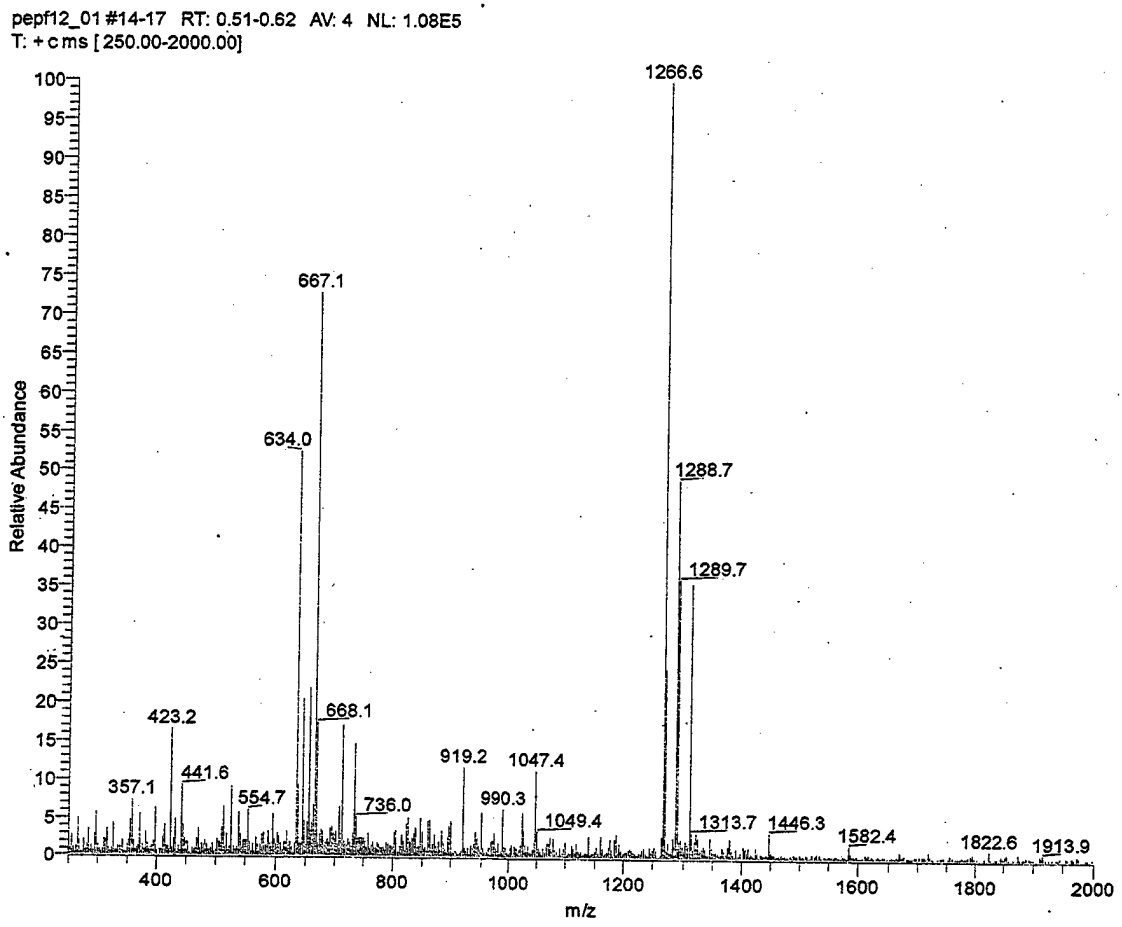

Figure 8. Direct infusion of Sample 12.

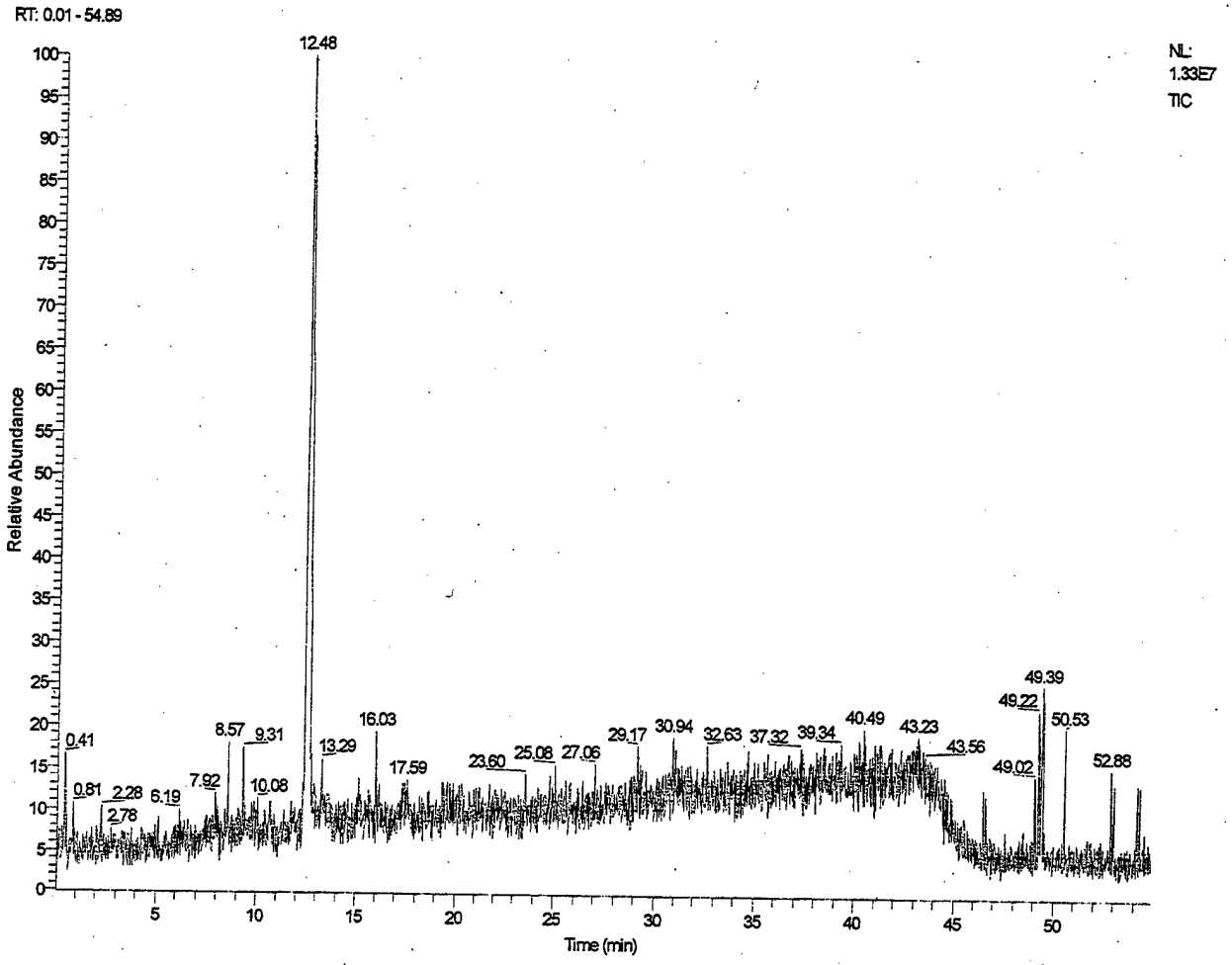

Figure 9. Total Ion Chromatogram of Sample 12 (Inset - Mass Spectra for RT 12.37 min -12.61 min.)

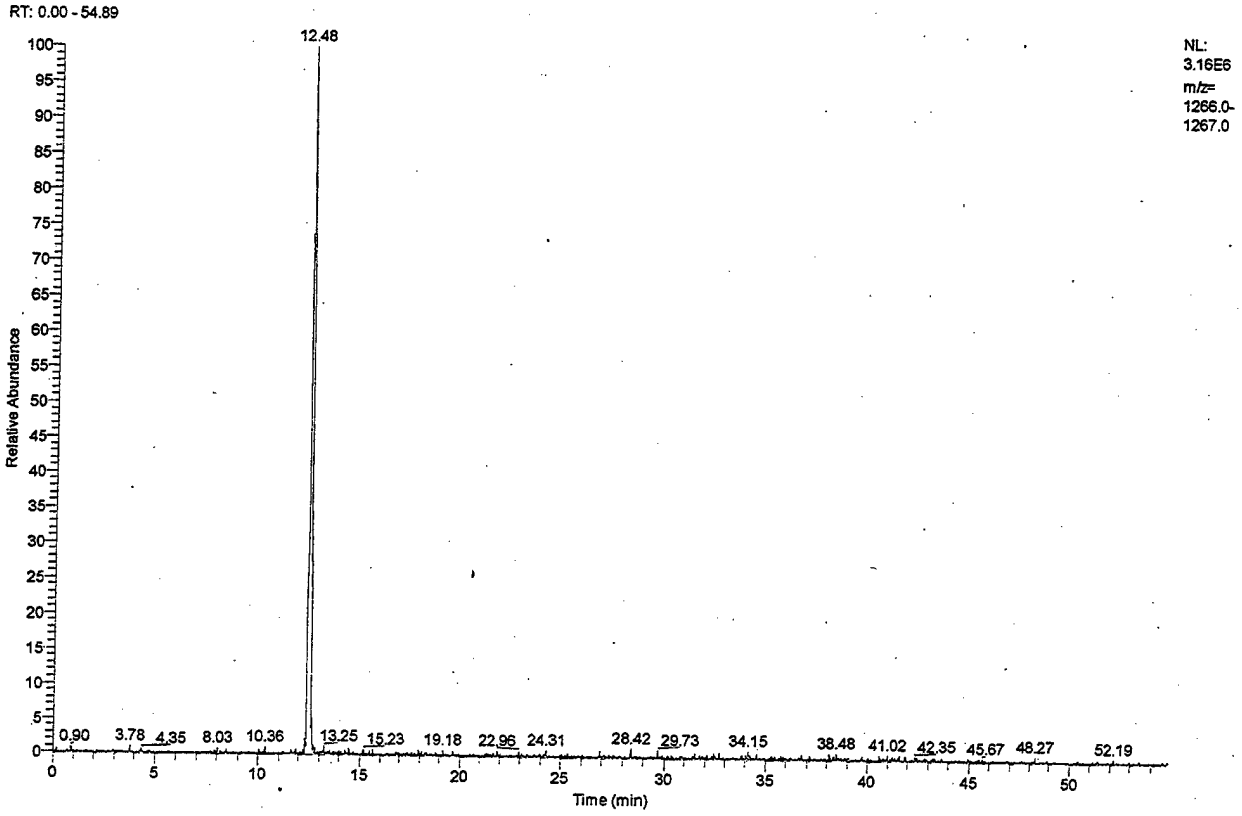

Figure 10. Extracted Ion Chromatogram for Sample 12.

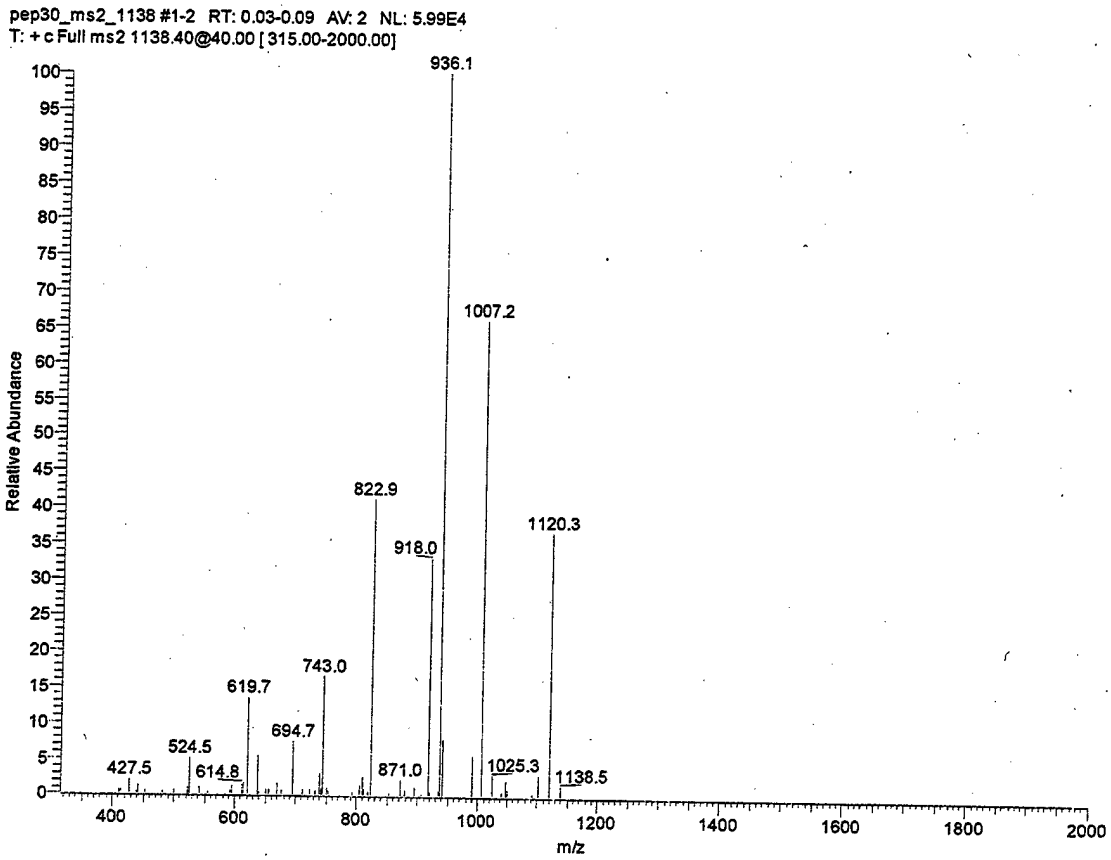

Figure 11. MS-MS of Sample 30, with peptide sequence (PVAKKIGKIAL).